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**THE ROLE OF IL-7 IN
LYMPHOPENIA AND
BYSTANDER APOPTOSIS
DURING HIV-1 INFECTION**

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A piece of the puzzle...

ABSTRACT

The concentration of interleukin-7 (IL-7) in human serum is elevated in various clinical conditions associated with lymphopenia. IL-7 is an essential factor for T cell differentiation and survival, and high IL-7 concentration has been proposed to represent a homeostatic response to T cell depletion, which may accelerate thymic output and promote T cell regeneration. During HIV-1 infection, however, high levels of IL-7 are correlated with CD4+ T cell depletion and appear not to be beneficial to rescue the diminishing T cell pool.

In order to further understand the impact of T cell numbers on serum IL-7 levels at different clinical stages of HIV-1 infection we investigated specimens from HIV-1 infected patients during primary and chronic infection and in long term non-progressors (LTNPs). In patients with primary HIV-1 infection, CD4+ and CD8+ T cell counts showed no correlation with the high IL-7 levels found in these patients; however the significant association seen between IL-7 and total CD3+ T cell counts may reflect an effect of lymphopenia on the increased IL-7 level, as previously reported in chronic HIV-1 infection. We also studied IL-7 levels in LTNPs, characterized by CD4+ T cell counts above 500 cells/ μ l and control of viral replication for 7 to 10 years without ART. Some of the LTNPs individuals progressed to a symptomatic phase of HIV-1 infection and, interestingly, we observed that these individuals showed a higher IL-7 level before progression as compared to the LTNPs that maintained high CD4+ T cell counts and virological control.

We asked the question on whether positive effects of IL-7 on survival and homeostatic proliferation of T cells might be severely impaired in HIV-infected individuals due to IL-7R α down-regulation. Thus the frequency of IL-7R α - T cells in HIV-1 infected patients was studied in relation to CD4 T-cell counts, IL-7 concentration and expression in different T-cell populations. Down-regulation of IL-7R α on T cells correlated with depletion of CD4 T cells ($P < 0.001$) and also with increased concentration of serum IL-7 ($P < 0.05$). Particularly, T cells with memory phenotype showed a decreased IL-7R α expression in association with CD28 down-regulation. Thus, IL-7R α down-regulation and differentiation towards a CD28- memory phenotype in response to chronic activation may lead to an overall decrease of IL-7 mediated survival within the peripheral T-cell pool.

The loss of CD4+ T cells during HIV-1 infection is not entirely the cause of direct infection of these cells, but is also due to bystander apoptosis where uninfected cells are predisposed to death inducing signals. As elevated IL-7 levels occur in HIV-infected individuals in addition to high Fas expression on T cells and increased sensitivity to Fas-induced apoptosis, we analyzed whether IL-7 has a regulatory role in Fas-mediated T cell apoptosis. We showed that IL-7 up-regulates *in vitro* Fas expression on naïve and memory T cells through a mechanism that involves translocation of Fas molecules from intracellular compartments to the cell membrane. The role of IL-7 in Fas upregulation *in vivo* was verified in IL-7 treated macaques. In addition IL-7 treatment primed T cells for Fas-induced apoptosis *in vitro* and serum IL-7 levels correlated with the sensitivity of T cells to Fas-induced apoptosis in HIV-infected individuals. Our data suggest that elevated IL-7 levels associated with HIV-1 infection, might participate in the increased sensitivity of T cells for activation-induced apoptosis.

Alteration of receptor-mediated apoptosis is not limited to HIV-1 infection, but is also present in other infections including Leishmaniasis. Leishmaniasis infections often occur in areas of high HIV-1 prevalence. During Cutaneous Leishmaniasis (CL), caused by *L. Major* infection, there is a chronic inflammation process that leads to killing of the non-infected keratinocytes in the epidermis followed by disfiguring scar formations. Our studies showed that the expression of Fas, TRAIL-R2 and TRAIL is increased on keratinocytes upon exposure to supernatant from *Leishmania* infected PBMC cultures and in diseased skin from patients with CL. The expression of death receptors also renders the keratinocytes more sensitive to apoptosis and they can die through a bystander effect due to infiltrating immune cells expressing death ligands. Blocking Fas and TRAIL *in vitro* inhibits, to a great extent, apoptosis occurring in the experimental procedure.

LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following original papers and manuscripts that will be referred to in the text by their roman numerals:

- I. **Fluur C**, Rethi B, Thang PH, Vivar N, Mowafi F, Lopalco L, Uberti Foppa C, Karlsson A, Tambussi G, Chiodi F. *Relationship of interleukin-7 levels and lymphopenia in early and late stages of HIV infection*. (submitted)
- II. Rethi B, **Fluur C**, Atlas A, Krzyzowska M, Mowafi F, Grutzmeier S, De Milito A, Bellocco R, Falk KI, Rajnavolgyi E, Chiodi F. *Loss of IL-7Ra is associated with CD4 T cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients* AIDS. 2005 Dec 2;19(18):2077-86.
- III. **Fluur C**, De Milito A, Fry TJ, Eidsmo L, Atlas A, Federici C, Matarrese P, Logozzi A, Rajnavölgyi E, Mackall CL, Fais S, Chiodi F and Rethi B. *Possible role for IL-7 in Fas mediated T cell apoptosis during HIV-1 infection* (submitted)
- IV. Eidsmo L*, **Fluur C*** (first share authorship) , Rethi B, Eriksson Ygberg S, Ruffin N, De Milito A, Akuffo H and Chiodi F. *FasL and TRAIL induced epidermal apoptosis and skin ulceration upon exposure to Leishmania Major* American Journal of Pathology. 2007 170:227-239

LIST OF ABBREVIATIONS

AICD	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
AIP	Apoptosis inhibiting protein
Apaf-1	Apoptosis protease-activating factor-1
ARS	Acute retroviral syndrome
ART	Antiretroviral therapy
AZT	Zidovudine
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FADD	Fas associated death domain
FasL	Fas ligand
FRET	Fluorescence resonance energy transfer
HEK	Human epidermal keratinocytes
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN- γ	Interferon-gamma
LTNP	Long-term non-progressor
MBL	Mannose binding protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NK	Natural killer
NNRTI	Non-nucleoside analog reverse transcriptase inhibitor
NRTI	Nucleoside analog reverse transcriptase inhibitor
PI	Protease inhibitor
RTE	Recent thymic emigrants
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TGF- β	Transforming growth factor-beta
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis inducing ligand
TSLP	Thymic stromal lymphopoietin

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1 INTRODUCTION

1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

In 1981, five young, previously healthy, homosexual men in Los Angeles were reported to be affected by an unusual clustering of rare diseases like Kaposi's sarcoma and *Pneumocystis carinii* pneumonia. They all had a common immunological dysfunction in that they presented with a significant reduction of circulating CD4+ T cells. In parallel, clinicians in Europe also started to observe similar symptoms among their patients and they established a group to study this emerging disease. Shortly after, the association between a retrovirus called HIV (initially called HTLV-III) and acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983) was established.

As of today more than 25 million people have died in AIDS, according to the UNAIDS/WHO report in 2006, and approximately 40 million people are living with HIV. About 5 million get infected and 3 million die of AIDS every year throughout the world. The most notable region of HIV prevalence is the sub-Saharan Africa, but the incidence is accelerating in some countries and regions including China, India and parts of eastern Europe and central Asia.

1.1.1 Transmission and pathogenesis

HIV is a retrovirus belonging to the family of lentiviruses which carry their genetic information in the form of RNA. Like all retro- and lentiviruses, HIV-1 must integrate into the DNA of the host cell. As a cause of this, the activity of the integrated viral genome is greatly influenced by the metabolic and activation state of the host cell, meaning that the longevity of the virus is dependent of the life span of the cell that it is integrated in. This is clearly seen in HIV-1 infection where a reservoir of the virus is established in the CD4+ T cells (reviewed in (Blankson et al., 1999)).

HIV-1 can be transmitted through blood transfusion and from mother to child, but is predominantly transmitted through heterosexual contacts where the virus crosses the mucosal barriers. The probability of infection for each encounter with HIV-1 is quite small and is partly related to the viral load of the transmitter.

Once the virus has made its way through the mucosal barrier it is bound by Langerhans cells and dendritic cells (DCs) that will transfer the virus to CD4+ T cells. The infected CD4+ T cells will make their way to the lymphoid tissue where the active replication and spread of the virus to other organs and peripheral tissues takes place, thus establishing tissue reservoirs. Transmission of HIV-1 to CD4+ T cells is mediated by interactions between viral surface proteins gp120 and gp41, the receptor CD4 and the chemokine coreceptors CCR5(Alkhatib et al., 1996) and CXCR4 (Deng et al., 1996). Different strains of the virus termed R5 and X4, bind the CCR5 and CXCR4 coreceptors respectively. Primarily R5 virus is transmitted, whereas the X4 strain occurs later during the infection and is a marker of a more rapid progression to AIDS (Schuitemaker et al., 1992). The importance of the CCR5 coreceptor in HIV pathogenesis was proven by the finding that cells from individuals with a 32bp deletion in the CCR5 gene can not be infected by R5

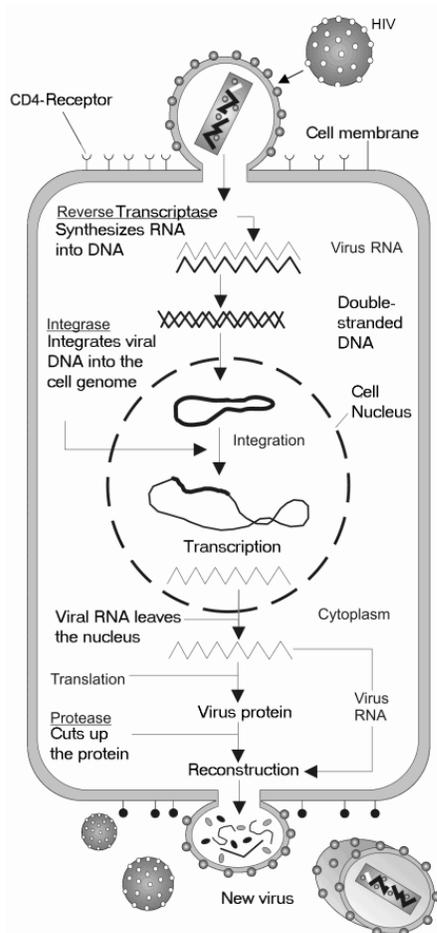


Figure 1. Replication cycle of HIV in a target cell. Adapted from Wikipedia online.

virus *in vitro*. About 1% of the white population who carry this deletion is extremely resistant to infection even when repetitively exposed to the virus (O'Brien and Moore, 2000). Once the virus has entered the cell, as briefly described in Figure 1, the viral RNA is transcribed into DNA by reverse transcriptase and then integrated into the cell genome. New viral RNA is transcribed that leaves the nucleus into the cytoplasm where it is translated into viral particles that are released through budding endosomes using the cells own membrane as a shield. Although the exact course of HIV-1 infection and onset of disease varies among different individuals, there is a general scheme for the progression to AIDS in untreated patients. In the first weeks of infection there is an acute phase where the viral

load rapidly becomes very high and the CD4+ T cells decline. During primary infection symptoms may occur consisting of fever, lymphadenopathy and rash. These symptoms which are collected under the name acute retroviral syndrome (ARS) disappears after a few weeks from primary infection. In the majority of infected individuals however, primary infection goes unnoticed. HIV-1 is most commonly detected by the presence of anti-HIV antibodies, although in the first three months there is no detectable virus or antibodies against HIV-1. Once antibodies are found in serum it is defined that the individual has seroconverted. After seroconversion the virus is kept in check by the immune system, the viral load is lower and the CD4+ T cell count is increased again. The infected individual is now in a chronic phase that can last for years with little or no clinical signs of disease. Eventually the virus breaks through the hosts immune defenses which results in the increase of viral load and loss of CD4+ T cells. When the amount of CD4+ T cells is as low as 200 cells/mm³ blood, the patient has progressed to AIDS. This course of infection is illustrated in Figure 2, where the CD4+ T cell count and viral RNA copies are associated with the time span of the disease.

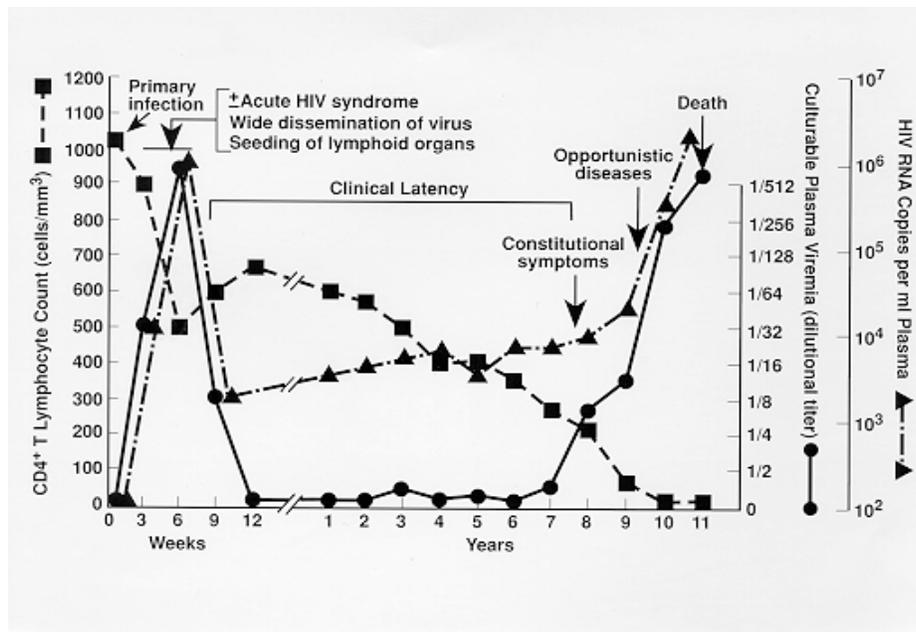


Figure 2. Time course from primary HIV-1 infection to the development of AIDS. The relationship between CD4+ T cell counts and viral load is illustrated together with the occurrence of symptoms and opportunistic diseases. Adapted from Wikipedia online

1.1.2 Immune response and escape

HIV-1 infection is essentially targeting the immune system and eventually makes it to collapse rendering it unable to fight opportunistic infections.

1.1.2.1 Innate response

As in other infections, the innate immunity is the first line of defense against HIV-1 infection, found at mucosal surfaces where the key cellular participants are DCs and macrophages. These cells present pattern recognition receptors called toll-like receptors (TLRs) on their surface that recognize specific ligands. The recognition of ligands initiates a cascade of events which eventually leads to the secretion of cytokines and various immune responses (Medzhitov and Janeway, 2000).

There are also soluble factors that can exert anti-HIV activities directly such as complement, defensins, cytokines, chemokines and lectin-binding proteins. The mannose-binding lectin (MBL) attaches to HIV-1 and can enhance its phagocytosis by macrophages, or in the presence of complement directly lyses the virus (Ezekowitz et al., 1989) (Saifuddin et al., 2000). Defect, or low levels, of MBL has been shown to be associated with an increased risk of HIV infection, rapid progression to AIDS and a shorter survival after AIDS onset (Garred et al., 1997). Complement alone can directly interact with HIV-1 to lyse the virion particles and constitutes another important soluble component of plasma (Sullivan et al., 1996). In essence, these soluble factors can attack the free virion and play important roles in the rapid response against HIV-1 infection.

1.1.2.2 Adaptive response

At the onset of the adaptive immune response, which occurs during the early phase of acute infection, there is a clear reduction of viral load an improvement of CD4+ T cell number and resolution of the ARS (Koup et al., 1994) (Rosenberg et al., 1997).

The adaptive response consists mainly of neutralizing antibodies and virus specific CD8+ cytotoxic T lymphocyte (CTL) responses.

Antibody responses

In many viral infections, antibody mediated clearance has a central role in eliminating the virus. This does not seem to be true for HIV-1 infection. Antibodies in the sera of HIV-1 infected patients have only a very weak neutralizing effect on primary HIV-1 isolates (Moore et al., 1995), where most of the antibodies are non-neutralizing and instead specific for viral debris (Poignard et al., 1999). Depletion of B cells in rhesus macaques, using CD20 directed antibodies, significantly postponed the emergence of a virus neutralizing antibody response after SIV infection. This did not modify the kinetics of early viral clearance, suggesting that neutralizing antibodies may not be important in the early control of the virus (Schmitz et al., 2003). Antibodies that do neutralize HIV-1 recognize different domains of the viral surface such as the third hypervariable loop (V3) of the envelope glycoprotein (Javaherian et al., 1989), the CD4 binding sites of the envelope (Reitter et al., 1998) and the transmembrane protein (Trkola et al., 1995). Trials where HIV-1 infected patients have been infused intravenously with preparation of immunoglobulins containing high titers of HIV-1-specific antibodies did not show significant effect on viral load or disease progression (Jacobson, 1998). On the other hand, neutralizing antibodies pre-administered before infection with SIV and SHIV in macaques have been shown to alter the clinical outcome (Mascola et al., 2000) (Baba et al., 2000). The observation that neutralizing antibodies fully protect against initial infection suggests that such antibodies will be of outmost importance in any strategy to prevent HIV-1 infection.

Cellular response

In contrast to the observations about neutralizing antibodies, virus-specific CTLs have been found to be clearly involved in the control of HIV-1 replication. CTLs specific for HIV-1 have been located in large numbers in a variety of anatomic compartments in both humans and macaques. Additionally, *in vitro* studies show that CD8⁺ T cells can inhibit HIV-1 replication (Walker et al., 1986), and lyse HIV-1 infected cells to block dissemination of the infection (Tsubota et al., 1989). The CTLs can also produce soluble factors that mediate lysis of target cells. These factors, MIP-1 α , MIP-1 β and RANTES, co-localize with granzymes and perforin and are secreted by CTLs after being triggered by antigen encounter (Wagner et al., 1998).

During the early phase after infection there is a partial control of the virus replication. An association between the appearance of effector cell populations that lyse HIV-1 infected target cells and a decline in plasma viral load during the primary HIV-1 infection has been shown (Koup et al., 1994) (Borrow et al., 1997). Probably the most convincing evidence for the importance of CD8+ CTLs in restraining the HIV-1 replication comes from studies in SIV infected rhesus macaques. Depletion of CD8+ T cells in monkeys *in vivo*, by infusion of monoclonal antibodies against CD8, had a marked effect on the replication of SIV. If the duration of depletion was greater than 28 days, primary viremia was never cleared and the monkeys rapidly progressed to an AIDS-like syndrome and died. In addition, if the CD8+ T cells were depleted in chronically SIV infected monkeys this led to a substantial increase of viral load that returned to baseline levels if the administration of the antibody was stopped and the CD8+ T cell population re-emerged (Schmitz et al., 1999) (Jin et al., 1999).

In concert with CTLs being important in controlling the virus replication, the major histocompatibility complex MHC class I presenting the viral antigens can also determine the outcome of a response. The particular fragment of a virus that is immunogenic for CTLs needs to be presented by the most optimal MHC I molecule to generate a potent CTL response. It is found that heterozygosity at class I alleles as well as the expression of MHC I molecules HLA-B27 and HLA-B57 in HIV-1 infected individuals is related with a better clinical outcome than if the MHC I molecule HLA-B35 would be expressed (Migueles et al., 2000) (Carrington et al., 1999) (Gao et al., 2001).

CD4+ T lymphocytes that are specific for HIV-1 also have a very central role in controlling HIV-1 replication since they facilitate CTL and antibody responses. Sensitive assays for measuring cytokine production by viral peptide-stimulated lymphocytes have shown that many HIV-1 infected patients have virus-specific CD4+ T lymphocyte populations (Pitcher et al., 1999) (McNeil et al., 2001). The magnitude of CD4+ T cell proliferation and cytokine production also correlate with the clinical status of HIV-1 infected individuals and SIV infected monkeys (Rosenberg et al., 1997) (McKay et al., 2003).

1.1.2.3 Immune escape and dysfunction of the immune system

There have been many suggestions on how HIV-1 manages to evade the immune system, but the best documented is through the generation of mutations in targeted epitopes of the virus. Its error-prone reverse transcriptase and high replication rate allow for rapid replacement of circulating virus by those carrying resistance mutations that can escape recognition by the immune system. Selection pressure applied by humoral and cellular immune responses to HIV-1 is well recognized, but its precise involvement in immune failure is still not fully understood.

Studies with recombinant virus assays have revealed that the rate of neutralizing antibody escape even exceeds the rapid rate of drug selection pressure and can account for the broad variability in the envelope proteins compared with other genes (Richman et al., 2003) (Wei et al., 2003). The mechanism of escape may involve changes in envelope glycans that shield antibody binding sites by steric hindrance (Wei et al., 2003).

Evasion of CTL responses have been documented both in acute (Borrow et al., 1997) (Price et al., 1997) and chronic (Phillips et al., 1991) (Goulder et al., 1997) HIV-1 infection. It is known that escape can occur even through single amino acid mutations in an epitope (Pelemans et al., 1997); these mutations are often placed at sites essential for MHC binding or T cell receptor recognition, but may also be located in flanking regions that effect antigen processing. In SIV infected monkeys strong initial CTL responses were generated against an epitope in Tat. Even though the infecting virus was controlled by an efficient CTL response against an early-expressed Tat epitope, new viruses with mutations in Tat emerged as this Tat-specific CTL response was being generated, and variant viruses went on to establish a chronic infection beyond control (Allen et al., 2000).

Since not all viral CTL epitopes generate escape mutations, there is in addition a functional impairment of cellular immune responses that is of benefit for the HIV-1 virus to maintain infection. There have been several suggestions on how this immune dysfunction is established. Animal studies of immune failure in chronic viral infections demonstrate that it is likely the lack of sufficient HIV-1-specific CD4+ T helper cell proliferation and expansion that serves as a crucial feature of this impairment (Pitcher et al., 1999) (McNeil et al., 2001). In

monkeys, there is a marked loss of the ability to express cytokines that begins early at the time of peak viremia in acute infection (McKay et al., 2003). Since HIV-1 selectively infects HIV-1-specific CD4+ T cells this provides a mechanistic explanation for the loss of these cells early in infection (Douek et al., 2002). There are other mechanisms that the virus uses for evading the immune system, but the importance of their involvement in the overall immune dysfunction is less evident. It has been noted that down-regulation of HLA class I by the viral Nef protein impairs CTL recognition (Collins et al., 1998), and this limits the inhibitory effects of CTLs on viral replication (Yang et al., 2002). Also, defects in differentiation and maturation of CTLs may result in impaired *in vivo* function and possibly relate to the lack of CD4+ T cell help (Champagne et al., 2001) (Appay et al., 2002). Other groups have found that CTLs against HIV-1 are lacking perforin or cytokine production (Zhang et al., 2003) (Kostense et al., 2002), which are key signaling molecules for T cell activation and costimulation, thus contributing to immune impairment (Trimble et al., 2000). CD8+ T cells from HIV-1 infected individuals can secrete IFN- γ , but in those patients who do not control viremia there is a defect in the ability of CD8+ T cells to proliferate in response to antigenic challenge (Migueles et al., 2002). Obviously, induction of apoptosis by bystander mechanisms in non-infected cells of the immune system, as seen during HIV-1 infection, is also a way of evading immune recognition; this process and the molecular players involved in this process are extensively discussed in this thesis.

1.1.3 Today's treatments and immunotherapy

1.1.3.1 Antiretroviral therapy

Modern drug development has directed the fast clinical progression seen during HIV-1 infection into a treatable, chronic infectious disease with which infected patients can lead a relatively healthy life for a long period of time. The target for antiretroviral therapy is to stop virus replication by inhibiting different viral components involved in the HIV replication cycle. Today there are four different classes of antiviral drugs. Zidovudine (AZT), the first drug on the market, is a nucleoside analog reverse transcriptase inhibitor (NRTI) (Ezzell, 1987). Failure in solely using this drug led to the discovery of protease

inhibitors (PIs) (Dorsey et al., 1994), (Vacca et al., 1994), and later even more NRTIs and PIs were approved which lead to combination therapies, or ART, used for treatment (Hammer et al., 1997) (Hirsch et al., 1999). Later drugs belonging to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and fusion inhibitors were approved and gave an even wider spectrum of drugs to be used either alone or in combination.

However, there still remain problems with the current therapy such as resistance, side effects, compliance and the viral reservoir that can not yet be eliminated. When treatment fails the most common reason is compliance, since motivation and understanding the importance of taking the drugs varies among individuals. These problems, together with the fact that effective antiretroviral therapy may not reach people with HIV-1 infection in developing countries due to cost and difficulties in delivery of the drugs, makes the discovery of new approaches to treatment indispensable.

1.1.3.2 Immunotherapy

Many approaches to ameliorate immune reconstitution in HIV-1 infection are currently under way, but yet the proof of principle that a clinical benefit can be achieved is lacking. At least four approaches have so far been tested with various results such as adoptive therapy, cytokine therapy, therapeutic vaccinations and a combination of ART and treatment interruptions to boost immune responses against the virus.

Adoptive therapy has been evaluated using both antibodies and cells. In non-human primates, infusion of cocktails of neutralizing monoclonal antibodies has lead to a marked protection from infection (Mascola et al., 1997). CTL's specific for HIV-1 have been infused and seem to home to sites of viral replication (Brodie et al., 1999), and infusion into an AIDS patient rapidly selected escape mutants providing evidence for *in vivo* functions of CTL's (Koenig et al., 1995). Therapy with IL-2 infusion has resulted in clear increases in CD4+ T cell count (Kovacs et al., 1996), but still it is not clear whether this increase actually affects disease progression.

The design of new vaccines, prophylactic and therapeutic, is the focus of research around the world. By the use of a therapeutic HIV-1 vaccine, delivered to an immunocompetent, aviremic individual under ART, there is the

hope to boost the HIV-specific cell-mediated immunity, restrain viral replication and delay viral rebound. In the most optimal scenario this immunization would allow the patients to stay out of drug treatment for certain periods or theoretically to clear the viral reservoir. A prophylactic HIV vaccine can help controlling the spread of the HIV pandemic (Klein, 2003). To date, more than 35 vaccine candidates have been tested in clinical trials involving over 10,000 volunteers, but so far no vaccine has been proved successful. Several different approaches have been made in designing vaccines such as using live attenuated, inactivated, virus-like particles or pseudoviruses, subunit vaccines (envelope-based and non-structural), naked DNA and live recombinant vaccines, prime-boost combinations and also fusion proteins and peptides (McMichael and Hanke, 2003). Despite all these trials and 23 years of research the world is still waiting for a breakthrough in this area of research.

A temporary control of the virus has been achieved with early treatment followed by treatment interruptions in acute HIV-1 or SIV infection leading to a broadening and increased magnitude of cellular immune responses to the virus (Liszewicz et al., 1999). However, the same approaches have not been as successful in chronic HIV-1 infection (Oxenius et al., 2002) (Garcia et al., 2001). This is probably due to an increased variability of the virus during chronic infection and thus to a greater chance for immune escape, as well as a shortage of restoration of virus specific T helper cell responses with ART alone (Altfeld et al., 2001).

1.2 IL-7

Interleukin-7 (IL-7) was first discovered in 1988 as a factor that promoted growth of murine B cell precursors in the bone marrow (Namen et al., 1988). Later it became known as an essential cytokine for lymphoid development and survival, and a critical component of the lymphoid homeostasis.

1.2.1 Structure and the receptor

The human IL-7 is a glycoprotein of 25 kDa in its active form and although the crystal structure is yet to be determined the three-dimensional structure has

been predicted to show homology with other hematopoietins such as IL-2 and IL-4 (Cosenza et al., 1997; Kroemer et al., 1996). Induction of the synthesis of IL-7 is thought to be mediated by IFN- γ , where IFN- γ has been shown to up-regulate IL-7 mRNA expression in human intestinal epithelial cell lines (Oshima et al., 2004). Other cytokines such as IL-1 and TNF- α also induce IL-7 synthesis from human stromal cells and osteoblasts (Weitzmann et al., 2000), while TGF- β instead inhibits the IL-7 synthesis in stromal cells (Tang et al., 1997).

The IL-7 receptor belongs to the type I cytokine receptor family and consists of two parts. One part, called γ_c chain, is shared by other receptors of the same family that bind cytokines such as IL-2, IL-4, IL-9, IL-15 and IL-21, as illustrated by Figure 3. The other part, called IL-7 receptor α (IL-7R α), is almost unique in binding IL-7 apart from the thymic stromal lymphopoietin (TSLP), a protein acting mainly on dendritic cells and monocytes (Soumelis et al., 2002). Both chains are expressed independently on the surface of the cell, but pre-associate prior to binding the cytokine to generate a high affinity binding. This heterogenous association is essential for the biological effect of IL-7.

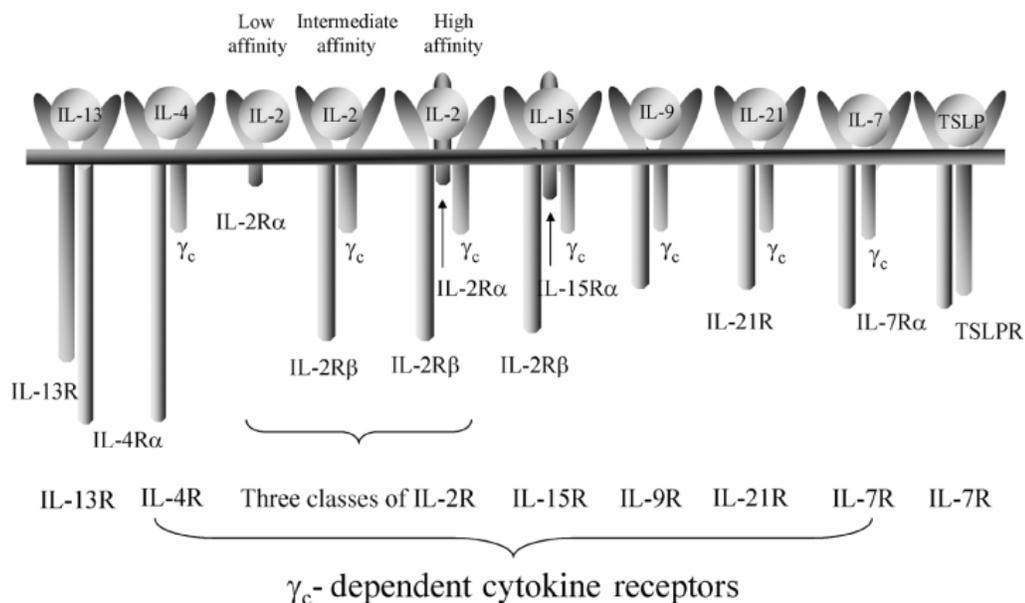


Figure 3. Illustration of receptors belonging to the type I cytokine receptor family sharing the γ_c chain. Adapted from Kovanen PE, *Immunol Rev* 2004

The signaling pathways induced by the binding of IL-7 to its receptor have been well studied and involve activation of the JAK/STAT pathway as well as the PI3-kinase pathway which all lead to increased expression of genes that promote lymphocyte survival and reduced expression of genes that induce lymphocyte apoptosis and cell cycle arrest (Benczik and Gaffen, 2004), (Khaled and Durum, 2003).

IL-7R α is mainly expressed on hematopoietic cells of the lymphoid lineage. These include fetal NK/dendritic cell precursors, the mutual T/NK/B cell lymphoid precursors, developing T and B cells, mature T cells and bone marrow macrophages. The level of the IL-7R α expression varies in lymphoid development and seems to be tightly regulated by mechanisms that have only been partly characterized (Xue et al., 2004). Soluble forms of IL-7R α have also been reported, both in healthy and leukemic hosts. These spliced variants of the receptor lack the transmembrane domain and a significant proportion of the intracellular domain, but they maintain the ability to bind IL-7 with a biological role that remains unclear (reviewed in (Sasson et al., 2006a)).

1.2.2 In development and in the mature immune system

IL-7 is an essential and non-redundant cytokine in the development of T cells. Its role in development is most clearly demonstrated by the rareness of lymphocytes present in IL-7- and IL-7R α -deficient mice and also following neutralization of IL-7 or its receptor *in vivo* (reviewed in (Fry and Mackall, 2005)). In the thymus, the earliest stem cells require IL-7 for survival, proliferation and rearrangement of some T-cell receptor genes. IL-7R α expression is lost however for a short critical time point when the cells need to proceed to a double positive stage; if not IL-7 inhibits important transcription factors that promote this process (Yu et al., 2004). At later stages of development, IL-7 is involved in the positive selection of CD8⁺ T cells (Khaled and Durum, 2002). Thus in early T cell development IL-7 signals are critical for survival, proliferation and rearrangement of genes, but loss of IL-7R α expression allow normal T cell development to proceed and may also help to maintain a sufficient double negative pool size.

Also, IL-7 secreted by stromal cells in the bone marrow drives the maturation of pro-B cells to pre-B cells. Pre-B cells expressing the IL-7 receptor no longer

need a direct contact with the stromal cells but continue to require IL-7 for growth and maturation (Richard Goldsby, 2003).

In the mature immune system naïve and memory T cells require IL-7 for survival and maintenance, since IL-7 induces up-regulation of survival factors such as Bcl-2 (Akashi et al., 1997; Maraskovsky et al., 1997), Bcl-xl and MCL (Opferman et al., 2003; von Freeden-Jeffry et al., 1997). Unlike other γc receptor cytokines, which are induced by immune responses, IL-7 is constantly produced at low levels that are detectable in human serum (Napolitano et al., 2001). During antigen stimulation and activation of naïve cells however, the T cells down-regulate the IL-7R α and thus have no need for IL-7. Instead they require IL-2 and other γc cytokines such as IL-4, IL-15 and IL-21 that are then produced in high amounts to boost the activation and proliferation. There is nevertheless heterogeneity in the down regulation of the receptor during an antigen specific immune response where a small number of the activated CD8⁺ T cells keep their IL-7R α expression. These CD8⁺ T cells are predestined to become memory cells after the immune response is terminated (Kaech et al., 2003). A transient down-regulation of the receptor on resting T cells is also demonstrated to occur after IL-7 binding (Paper II in this thesis). This suggests the interesting possibility that a feed back control loop may regulate the receptor expression in that cells already given the IL-7 signal will not compete for the cytokine, ensuring that other cells will be provided with the cytokine as well.

1.2.3 Production and T cell homeostasis

Several cell types in humans have been identified to produce IL-7. Intestinal epithelial cells (Watanabe et al., 1995), keratinocytes (Heufler et al., 1993), hepatic tissue (Golden-Mason et al., 2001), cells in peripheral blood and follicular dendritic cells (Sorg et al., 1998) (Kroncke et al., 1996), endothelial cells, smooth muscle cells, fibroblasts and stromal cells of the bone marrow and thymus (Kroncke et al., 1996), are all shown to produce IL-7. However, yet little is understood regarding its production in vivo. It is not at all obvious what cells produce IL-7 during physiological and pathological conditions and it still

remains to be investigated whether the synthesis of the cytokine by these cells is regulated or constitutively produced.

The peripheral T cell pool has a constant size. Even though there is an ongoing production of new T cells from the thymus this means that some others in turn have to die. In addition to thymic output, peripheral T cell populations also expand through proliferation (Almeida et al., 2005). Animal models suggest that IL-7 improves immune reconstitution through increasing thymic output and, perhaps more importantly, through antigen-independent proliferation in the periphery (Goldrath et al., 2004). This turnover, or cycling of T cells occurs constantly in lymphoreplete hosts where basal levels of IL-7 primarily mediate survival and low-level cycling of recent thymic emigrants (RTEs). IL-7 may also contribute to the modest cycling of memory populations that occurs throughout life. In addition IL-7 plays a role in the generation of memory cells in response to associated antigens, but this is not entirely dependent on IL-7. The homeostatic cycling of naïve cells takes part in the presence of IL-7 and low affinity antigens, whereas the cycling of the memory cell population occurs with IL-7 alone and without TCR stimulation (reviewed in (Fry and Mackall, 2005)).

During acute and chronic infections the picture is slightly different when it comes to memory formation and composition of memory subsets. After an acute infection the memory T cells survive in the absence of antigen. However, in an unresolved chronic infection there is need for constant antigen stimulation to maintain the memory T cell pool. It has been shown that in acute infection the memory T cells are able to proliferate in an antigen-independent way in response to IL-7 and IL-15. In chronic infection the receptors for IL-7 and IL-15 are lost and the memory T cells are thus unable to proliferate in response to antigen-independent homeostatic signals (Wherry et al., 2004). A recent study shows that, by early treatment of HIV-1 infection, functionally competent antigen specific memory CD8⁺ T cells expressing high levels of IL-7R α are preserved (Sabbaj et al., 2007).

IL-7 has been best described as a homeostatic modulator during lymphopenic conditions where its levels in serum have been found to be exceedingly high. These high levels are largely responsible for inducing homeostatic peripheral expansion in response to the small number of lymphocytes. Several *in vivo* experiments have been performed where IL-7 have been administered. In

lymphopenic mice there is an increase in thymic emigrants and also in T and B cells following injection of IL-7 (Morrissey et al., 1991), and in non-human primates injection of IL-7 increases the amount of circulating lymphocytes in normal and SIV infected hosts (Fry et al., 2003). A recent study also reveals that IL-7 administration in humans leads to an expansion of peripheral T cells suggesting an important role for IL-7 in the treatment of patients with lymphopenia (Rosenberg et al., 2006). Another possibility of using IL-7 in immunotherapy is as a vaccine adjuvant. IL-7 could then be given together with an immunogenic HIV vaccine aiming at inducing CTL's and promote their survival, proliferation of antigen-specific cells and help to establish a pool of memory T cells. However, a recent vaccination trial in combination with IL-7 administration was a disappointment, in that there was no increase in memory responses to a peptide vaccine (Rosenberg et al., 2006).

1.2.4 In HIV and other lymphopenic conditions

Infection by HIV-1 leads to severe T cell lymphopenia and general immune dysfunction. Both adults and children infected with the virus show higher levels of IL-7 in serum than the healthy population, and this inversely correlates with the level of CD4+ T cells (Llano et al., 2001). Higher IL-7 levels are associated with depletion of both memory/effector and naïve T cells, and also correlate with a higher viral load. Patients who respond well to ART treatment with an increase in their CD4+ T cell count also show a reduced level of IL-7; even in treated individuals however the level of IL-7 never return to the level found in non-infected individuals (Beq et al., 2004).

A similar association between CD4+ T cell count and serum IL-7 level has been found in other lymphopenic conditions such as patients undergoing cancer chemotherapy or bone marrow transplantation (Fry et al., 2001) (Bolotin et al., 1999). The increased concentration of IL-7 noticeable in T cell depleted individuals is thought to be a homeostatic response to the diminished T cell pool, which in turn may accelerate thymic output and promote peripheral T cell survival and proliferation (Fry et al., 2001) During lymphopenic conditions the level of IL-7 is raised (Fry and Mackall, 2005), but it is not yet clear whether this raise is due to less consumption by the diminished T cell pool, or if it is due to a

higher production of the cytokine. This is a very important question that remains to be solved.

1.3 APOPTOSIS

In an article published in 1972, John F. Kerr, Andrew H. Wyllie and A. R. Currie, coined the term "apoptosis" in order to differentiate naturally-occurring developmental cell death, from the necrosis that results from acute tissue injury (Kerr et al., 1972). The phenomenon of apoptosis was first known to be involved in embryogenesis shaping the digits of a fetus by removing the cells in between.

1.3.1 General concept

Apoptosis was later on found to take part in many other physiological processes used by an organism to selectively eliminate cells that are no longer needed. There is a constant removal of senescent cells that have already completed their job and of cells that are of danger to the organism such as cancerous, hyperactivated, autoimmune or infected cells. These events protect the organism and maintain the homeostasis of cells, such as blood and skin cells, that are being constantly produced. In the immune system, apoptosis plays an important role in several processes of immune function; in the development of T and B cell maturation, in regulation and termination of immune responses and in cell-mediated cytotoxicity (Strasser and Bouillet, 2003) (Krammer, 2000).

In cells undergoing apoptosis the protein synthesis is stopped, the mitochondrion becomes dysfunctional and chromatin condensation and DNA fragmentation is initiated. The cell shrinks and the cell membrane changes its protein expression to facilitate for phagocytosis. Typical blebbings are seen, before the cell is broken up into smaller apoptotic bodies (Ziegler and Groscurth, 2004). During the whole process the cell membrane is intact, preventing intra-cellular contents to leak out into the surrounding tissue causing inflammation.

Apoptosis occurs either in a receptor-dependent way called the extrinsic pathway, or in a non-receptor-dependent way termed the intrinsic pathway. In the extrinsic pathway a death receptor binds its ligand to initiate apoptosis, as it will be extensively described later. The intrinsic pathway is induced via the mitochondrion by UV-irradiation, DNA damage, granzymes and other intracellular damaging agents. Both pathways, however, lead to the cleavage of a group of cysteine proteases called caspases that catalyse a cascade of molecular events resulting in the activation or inactivation of numerous cellular proteins leading to the morphological changes described above. The response to death signals varies depending on cell type, developmental and activation stage of the cell, and the chemical or physical milieu, where all are regulated by the levels of anti-apoptotic and pro-apoptotic molecules.

1.3.2 Death receptor mediated apoptosis

The extrinsic pathway of apoptosis is activated through death receptors of several kinds. To date there are eight identified receptors which all belong to the TNF receptor superfamily, and have in common an extracellular cysteine rich domain and an intracellular globular protein interaction domain called death domain (DD). The known receptors are; Tumor Necrosis Factor Receptor 1 (TNFR-1), Fas (CD95), Death Receptor 3 (DR3), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR-1), TRAILR-2, DR6, ectodysplasin A receptor (EDA-R) and nerve growth factor receptor (NGF-R) (Schmitz et al., 2000) (Bouralexis et al., 2005). The ligands for these receptors function in an autocrine or paracrine manner, and upon binding cause a trimerization of their respective receptors, which is required for apoptotic signaling. When the receptor is activated adapter proteins are recruited to the DD, which also contains a death effector domain (DED). The DED of the adapter protein interacts with another DED on the apoptosis initiator enzyme procaspase-8 which is recruited into the death inducing signaling complex (DISC). The procaspase-8 is proteolytically cleaved into active caspase-8 and is released from the complex into the cytoplasm. Active caspase-8 cleaves various proteins in the cell, including procaspase-3 that becomes activated and completes the cell death programme (Sheikh and Fornace, 2000) (Daniel et al., 2001).

Belonging to the TNF family, there are also four receptors lacking the DD called decoy receptors. They are unable to transmit an apoptotic signal and are competing for death ligands with the death receptors. The decoy receptors that have been described are: TRAIL-R3, TRAIL-R4, DcR3 and osteoprotegrin (OPG) (Sheikh and Fornace, 2000). In this thesis the focus is on Fas and TRAIL-R1 and -R2 that have been investigated in the context of HIV-1 and Leishmania infections.

1.3.2.1 Fas-mediated apoptosis

Fas is a widely expressed surface molecule of type I transmembrane receptors. Its expression can be boosted by cytokines such as IFN- γ and TNF, but is also up-regulated upon activation of lymphocytes. Fas is only functional when assembled into a homotrimer, which occurs when its natural ligand, FasL, is bound. FasL belongs to the family of type II transmembrane molecules and is expressed in a much more restricted way than its receptor. Cytotoxic T cells and natural killer (NK) cells express and store FasL in intracellular vesicles, to ensure that high levels of membrane bound FasL are available during cytotoxic activity (Kojima et al., 2002). The ligand can also be cleaved off from the surface by metalloproteinases (MMPs), like MMP-7 (Vargo-Gogola et al., 2002). The soluble cleaved form of FasL also functions to induce apoptosis of Fas expressing target cells.

When Fas is bound by its ligand a complex of proteins are rapidly assembled to form the DISC (Kischkel et al., 1995), as previously described. The adapter protein in Fas mediated signaling is called FADD (Fas associated death domain), and also contains a DED domain. The signaling process events and involved molecules are otherwise the same as described for death receptors (Muzio et al., 1996).

Fas signaling can induce two types of intracellular pathways of which both lead to apoptosis (Scaffidi et al., 1998). In type I cells, the apoptotic cascade is propagated with DISC formation and cleavage and the activation of caspase-8. In type II cells however, the DISC formation is very weak and caspase-8 signaling needs to be amplified through the cleavage of the bcl-2 family member Bid. The truncated Bid induces cytochrome c release from the mitochondria. Cytochrome c together with the apoptosis protease-activating

factor 1 (Apaf-1) and the cytosolic procaspase-9 form a second apoptotic complex called the apoptosome, which will cleave and activate procaspase-3. The explanation for the need of having two different pathways initiated by death receptors is still unclear, but possibly there is a biochemical difference at the level of receptor activation.

For Fas mediated apoptosis to occur, there is a requirement for cytoskeletal changes. Ezrin, radixin and moesin all belong to a family of proteins involved in the linking of transmembrane proteins to the actin cytoskeleton (Fais et al., 2000). Connecting to actin is a crucial step in Fas-mediated apoptosis, both in predisposing T cells to Fas-mediated apoptosis, and in allowing the early steps of Fas signaling. It has been shown that T cells need to polarize their Fas molecules, with the help of ezrin-mediated Fas/actin association, to render them susceptible to Fas-mediated apoptosis (Luciani et al., 2004). Using an *in vitro* model where resting CD4+ T are exposed to HIV-1-gp120, it has been shown that association of Fas and ezrin predisposes cells to Fas-mediated apoptosis.

1.3.2.2 TRAIL mediated apoptosis

TRAIL, which is a relatively newly discovered molecule, belonging to the TNF family of proteins, induces apoptosis of TRAIL-R expressing cells in much the same way as FasL induced apoptosis. The soluble form of TRAIL, which is the most abundant form, is cleaved off TRAIL expressing cells by proteolytic enzymes. The receptors for TRAIL are TRAIL-R1, -R2, -R3 and -R4, where only TRAIL-R1 and -R2 bear the DD and are thus able of generating DISC formation and caspase-8 activation, inducing apoptosis either through a type I or type II cascade of events. TRAIL-R3 and -R4 are called decoy receptors as they are lacking the DD domain and are therefore unable to transmit any apoptotic signal. All these receptors are expressed in various amounts on the surface of cells, meaning that if the TRAIL-R1 or -R2 are overrepresented there will be apoptosis induced by the binding of their ligand TRAIL. On the other hand if TRAIL-R3 or -R4 is expressed in higher amounts, apoptosis will not occur. Except for being anti-apoptotic receptors, no other role for the decoy receptors is known (LeBlanc and Ashkenazi, 2003) (Bhardwaj and Aggarwal, 2003).

TRAIL is expressed in a variety of tissues, but its biological role is yet still not understood. The capacity of TRAIL to trigger apoptosis in a number of transformed cell lines suggests that it may be a modulator of tumor cell apoptosis (Ashkenazi et al., 1999) (Shankar and Srivastava, 2004). Increasing data points towards TRAIL being an important player in immune protection against oncogenic transformation and virally infected cells (Kelley and Ashkenazi, 2004).

Along the cascade of events that occur due to Fas or TRAIL signaling there are a number of anti-apoptotic molecules that prevent apoptosis to happen. FLIP, for instance binds the DISC and thus blocks the binding for procaspase-8. The AIP family of proteins can suppress apoptosis by interacting with, and inhibiting the enzymatic cleavage of caspases. Last but not least, members of the Bcl-2 family are fundamental regulators of apoptosis and are thought to act primarily on the mitochondria, preventing disruption of the membrane and release of cytochrome c.

1.3.3 Apoptosis in HIV and leishmania infections

Although apoptosis is meant to be a physiological regulator during an immune response against infection, the apoptotic system might be hijacked by the microorganism for pathogenesis and disease induction.

1.3.3.1 HIV

In HIV-1 infection, disease progression has been correlated both with higher viral load (Furtado et al., 1995) and with higher levels of lymphocyte apoptosis and activation (Gougeon et al., 1996). Even if HIV-1 can kill infected cells directly or through HIV-specific CTLs, apoptosis occurs mostly in non-infected cells. This was first shown by direct evidence in lymph nodes from HIV-1 infected patients where apoptosis was seen mainly in the uninfected bystander cells (Finkel et al., 1995). The uninfected cells can be killed by two different mechanisms; either by HIV proteins released by neighboring infected cells or by activation-induced cell death (AICD).

Discharged HIV proteins, or inactivated free HIV virions, in the extracellular environment can have dramatic effects on uninfected cells. The soluble HIV protein gp120 induces apoptosis in T helper cells through cross-linking of the

CD4 molecule (Banda et al., 1992). Soluble and also membrane bound gp120 induce apoptosis through cell receptors such as CD4, CXCR4 and CCR5 by a Fas-dependent (Fas/FasL up-regulation and decreased FLIP) or Fas-independent (more apoptotic Bax and less Bcl-2) ways (Arthos et al., 2002). In addition, the HIV proteins Tat, Nef and Vpr, secreted by infected cells, kill bystander cells that endocytose them by changing their expression of caspase-8 and FasL (Tat), or insert themselves into the plasma cell membrane causing membrane disruption (Nef and Vpr) (reviewed in (Alimonti et al., 2003)).

AICD is largely regulated by the Fas pathway and several studies demonstrate that Fas/FasL are up-regulated during the chronic phase of HIV infection and may therefore be involved in the destruction of T cells. Raised levels of both soluble Fas and FasL are found in HIV infected individuals and have been shown to be associated with disease progression (Medrano et al., 1998) (Silvestris et al., 1998). Macrophages expressing FasL can selectively kill uninfected T cells (Badley et al., 1996) (Herbein et al., 1998) susceptible for Fas mediated apoptosis in an MHC unrestricted manner (Badley et al., 1997), and a correlation is found between FasL expressing macrophages and the amount of lymphocyte apoptosis in lymph nodes of HIV infected patients (Dockrell et al., 1998). Likely, close cell to cell contact with FasL can induce apoptosis of Fas expressing cells (Tateyama et al., 2000). CTLs specific for HIV protein Nef can, in addition to using the perforin pathway against infected cells, also mediate killing through Fas/FasL, by targeting activated, non-infected Fas expressing lymphocytes (Garcia et al., 1997). Membrane bound Fas and FasL is found to be increased on both CD4+ and CD8+ T cells in HIV infection and this reflects their susceptibility to Fas mediated apoptosis which can be used as a marker of disease progression (Katsikis et al., 1995) (Sloand et al., 1997) (Estaquier et al., 1996), Furthermore, the susceptibility of Fas mediated killing during HIV infection is not seen in the non-pathogenic model of chimpanzees infected with HIV (Gougeon et al., 1997), giving additional weight to the importance of Fas/FasL in HIV pathogenesis.

A recently published paper indicated that IL-7 amplifies the sensitivity of CD8+ and CD4+ T cells for Fas-mediated apoptosis induced by HIV-1 LAI (Lelievre et al., 2005). Up-regulation of Fas and apoptosis sensitivity under this condition is

mediated by the interaction of CXCR4 with Env. This paper provided, for the first time, a link between IL-7 and a possible detrimental effect on HIV-1 pathogenesis.

The TRAIL receptor pathway has also been shown to be involved in the apoptosis of uninfected CD4+ T cells during HIV-1 infection. Elevated levels of TRAIL and CD4+ T cells expressing TRAIL-R2 are found in untreated HIV-1 infected patients (Herbeuval et al., 2005b), and CD4+ and CD8+ T cells from HIV-1 infected patients are more sensitive to TRAIL-induced apoptosis *in vitro* as compared to healthy controls (Katsikis et al., 1997) (Jeremias et al., 1998).

1.3.3.2 *Leishmania*

In *Leishmania Major* infection, causing the disease cutaneous leishmaniasis, apoptosis plays an important role for clearance of the parasite. The role of Fas and FasL during experimental leishmaniasis and parasite clearance has been investigated in several experimental settings where the infected macrophages are killed through the Fas/FasL pathway (Chakour et al., 2003) (Conceicao-Silva et al., 1998) (Desbarats et al., 2000) (Huang et al., 1998) (Ribeiro-Gomes et al., 2005). The vigorous inflammatory reaction however, often leads to a chronic inflammatory state where constant shedding of uninfected keratinocytes at the site of the ulcer are seen. Keratinocytes upregulated Fas as a result of the inflammatory environment surrounding *L. Major* infected macrophages, and at the same time high amounts of soluble and membrane bound FasL are present in the microenvironment (Eidsmo et al., 2005).

2 AIMS OF THIS THESIS

The basis for this thesis is the finding that the decline of CD4⁺ T cells occurs during HIV-1 infection in presence of high levels of IL-7. This observation is peculiar in that IL-7 is considered to be a cytokine promoting T cell survival and regeneration during lymphopenic conditions. Also, it is known that the Fas apoptotic pathway is involved in the decline of HIV-1 infected and non-infected T cells. Thus, the specific aims of this thesis are

- To clarify the association between lymphopenia and regulation of IL-7 levels during HIV-1 infection and other lymphopenic conditions
- To investigate the expression of IL-7R α on subpopulations of T cells during HIV-1 infection
- To assess the participation of IL-7 in promoting Fas-mediated apoptosis
- To study the role of Fas and TRAIL pathways in bystander cell killing of keratinocytes in *L. Major* infection and Cutaneous Leishmaniasis

In the present thesis, the methodological part has been omitted since the methods used in this work have been described in detail in the enclosed articles.

3 RESULTS AND DISCUSSION PAPERS I-IV

3.1 HIGH IL-7 LEVELS IN HIV-1 INFECTION: RELATIONSHIP TO LYMPHOPENIA AND MODULATION OF RECEPTORS ON T CELLS

3.1.1 IL-7 and lymphopenia at different stages of HIV-1 infection (I)

Since IL-7 has been designated to be a homeostatic factor increasing the number of T cells during lymphopenic conditions, we sought to investigate its role during different stages of HIV-1 infection which are associated with different CD4+ T cell numbers. Since it is still not fully understood what exactly regulates the levels of IL-7 in HIV-1 infection, we studied if the degree of lymphopenia has a direct impact and can modify the levels of IL-7.

Patients with primary HIV-1 infection show a reduced number of CD4+ T cells that will increase after the infection is somewhat controlled either by the hosts own immune system and/or by treatment with ART. When measuring IL-7 serum levels at baseline we found them to be high in the majority of primary HIV-1 patients investigated, whereas the CD4+ T cell counts were at different levels showing no correlation with IL-7 at this time point. In addition, there was no correlation with CD8+ T cells alone; however the significant association seen with the total levels of CD3+ T cells still reflects an effect of lymphopenia on the increased IL-7 level, as previously seen in chronic infection(Napolitano et al., 2001) (Mastroianni et al., 2001) (Llano et al., 2001) (Fry et al., 2001) (Fig 4a). Also, in patients where the time of ARS was known, the IL-7 levels were decreasing with time, reflecting the recovery of T cell numbers although this correlation was not significant ($p=0,07$). It was also previously shown that IL-7 levels in primary HIV-1 infection were elevated and correlated with CD4+ T cell reconstitution (Sasson et al., 2006b); treatment with ART resulted in normalized plasma IL-7 level in patients with primary HIV-1 infection.

In our study, the CD4+ T cell counts increased in primary infected patients that had received ART therapy for 5 months (12 out of 14 patients), as predicted.

The IL-7 levels were instead increased during this period and thus did not appear to be regulated by the lymphopenia in this setting. However the baseline IL-7 values correlated with the CD4+ and CD3+ T cell recovery seen after 5 months of therapy (Fig. 4b and c). This possibly indicates that if IL-7 levels are high before the onset of ART therapy, there is a better recovery of the T cells than if the IL-7 levels are low. This observation may suggest that administration of IL-7 in primary infected patients prior to ART may be beneficial for T cell recovery.

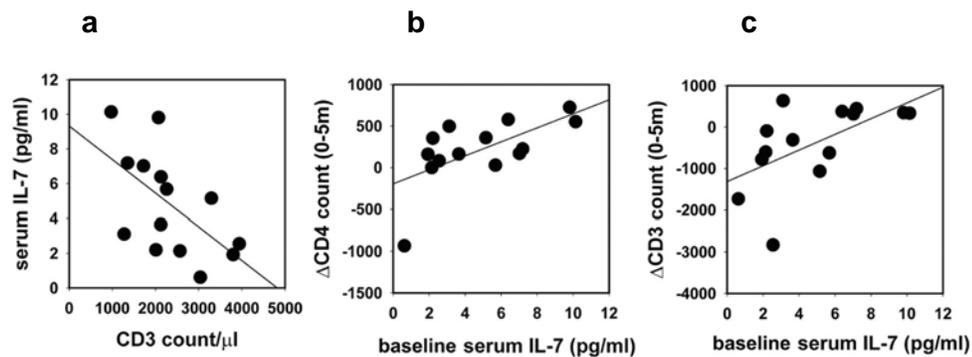


Figure 4. Correlations between serum IL-7 and T cell counts during primary HIV-1 infection. A cohort of 14 HIV-1 infected patients was analyzed at acute infection and 5 months later following ART administration. a) Serum IL-7 levels are compared to CD3+ T cell count. Changes in CD4+ T cell count (b) and CD3+ T cell count (c) during a 5 month period in correlation with levels of IL-7 at baseline are shown.

In chronic HIV infection a correlation has been observed with high IL-7 levels in patients experiencing a diverse treatment history with a CD4+ T cell count less than 200 cells/μl, and lower levels of IL-7 if the CD4+ T cell numbers exceed this amount. In order to exclude the influence of ART on the level of IL-7 we studied treatment naïve patients by measuring the IL-7 levels 2-4 times during a period of 13-56 months. In this setting we found no association between CD4+ T cell count and IL-7, but instead we did see a correlation between CD8+ and CD3+ T cells and IL-7 level indicating a possible role of lymphopenia in serum IL-7 regulation (Fig 5a and b). However, the changes observed in the T cell count and IL-7 levels did not correlate during this period of observation.

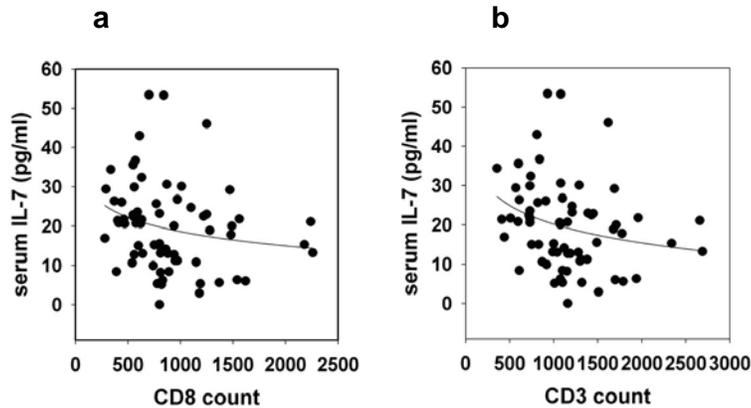


Figure 5. Correlation between T cell counts and IL-7 levels in chronic HIV-1 infection. Serum IL-7 levels were analyzed 2-4 times during a period of 13-56 months in a group of ART-naïve HIV-1 infected patients. a) serum IL-7 level is compared to CD8+ T cell count. b) serum IL-7 level is compared to CD3+ T cell count.

By investigating several, treatment naïve individuals during the same time period (13-56 months), and relating the changes in IL-7 levels and CD4+ and CD8+ T cell counts, we found a wide variation that appears to reflect individual differences. This suggests that during chronic HIV-1 infection, IL-7 levels only play a role in certain patients, perhaps in an immune system that is not yet exhausted and still can respond to the homeostatic pressure.

We studied IL-7 levels in LTNPs, characterized by CD4+ T cell counts above 500 cells/ μ l and control of viral replication for 7 to 10 years without ART, and we compared this group of asymptomatic individuals to chronically HIV-1 infected patients on ART. There was no difference in the IL-7 levels between the two groups. In some of the LTNPs investigated eventually the CD4+ T cell counts decline under 500 cells/ μ l and we made the interesting observation that these individuals showed a higher level of IL-7 before their progression as compared to the LTNPs that kept their high CD4+ T cell count and remained healthy (Fig 6). This finding could perhaps be of use as a prognostic value in the clinic, although that requires a more extensive study to be performed in order to generate a steady cut-off value given that the method of detection is stable. More likely, to follow variations of IL-7 levels in individual patients over time may help to predict the loss of LTNP state. The interesting question in this context is of course why it is not the contrary namely that patients who

progress should have lower IL-7 levels, since the IL-7 is a survival factor for T cells and should keep their number stable. Some other groups have shown on the other hand that IL-7 could increase viral replication from reservoirs and thus increase the viral load (Wang et al., 2005) (Smithgall et al., 1996); thus elevated levels of IL-7 may lead to increased viral replication and loss of CD4+ T cells. It might also be so that the remaining cells somewhat are defective in responding to IL-7, due to down-regulation of the IL-7R α (paper II), and by that way less IL-7 is used and IL-7 levels accumulate in body fluids. That this may be the case has been recently shown by the work of Sasson and collaborators (Sasson et al., 2006b) showing that changes in CD4+ T cell expression of IL-7R components were evident in patients with LTNP who lost viral control.

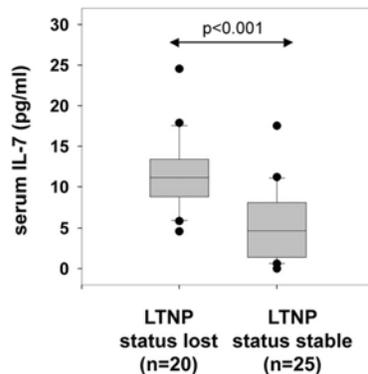


Figure 6. *LTNPs that loose the LNTN status have higher levels of IL-7. Serum IL-7 levels measured in a cohort of LTNPs that progressed with CD4+ T cell decline as compared to LTNPs that remained immunological and virologically stable.*

3.1.2 The regulation of IL-7R α in HIV-1 infection (II)

In a healthy individual there are about 15% of the T cells lacking the IL-7R α . These IL-7R α negative cells possibly reflect activated T cells with up-regulated IL-2R promoting T cell survival and proliferation. During HIV-1 infection, on the other hand, where there is a chronic activation of the immune system, the loss of the receptor can be found in between 30-60% of the T cells. The loss of IL-7R α in the majority of T cells in HIV-1 infection can be associated with disease progression since the amount of cells lacking the receptor is higher in patients with lower CD4+ T cell counts (Fig 7). This association could not be seen with CD8+ T cells or with viral load, although viral load and IL-7R α expression correlation pointed towards the same direction ($p=0,07$). ART treatment did not

influence the expression of the receptor since no difference in the loss of IL-7R α between ART treated and non-treated patients was observed.

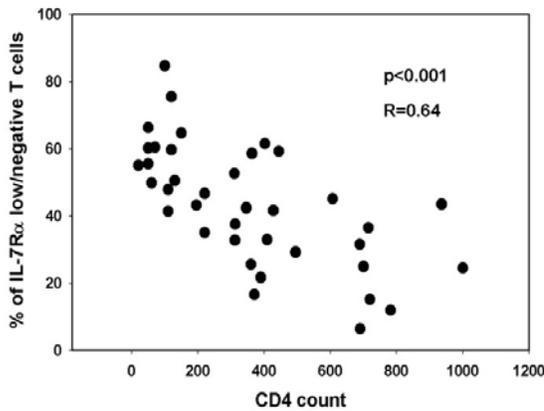


Figure 7. Loss of IL-7R α is correlated with loss of CD4+ T cells during HIV-1 infection. IL-7R α was measured on T cells from HIV-1 infected patients and correlated with their CD4+ T cell count in blood.

T cells from HIV-1 infected patients have a survival disadvantage in responding to IL-7 in culture due to the lack of receptor and a higher quantity of apoptotic T cells can be detected after 7 days in cultures with IL-7. T cells can only be rescued down to 40% apoptosis in cultures established with blood samples from HIV-infected patients, while in blood samples from non-infected controls only about 10% of T cells are apoptotic in cultures with IL-7 (Fig 8). The survival limitation was also verified by the measurement of less Bcl-2 expression in HIV-1 infected subjects as compared to controls.

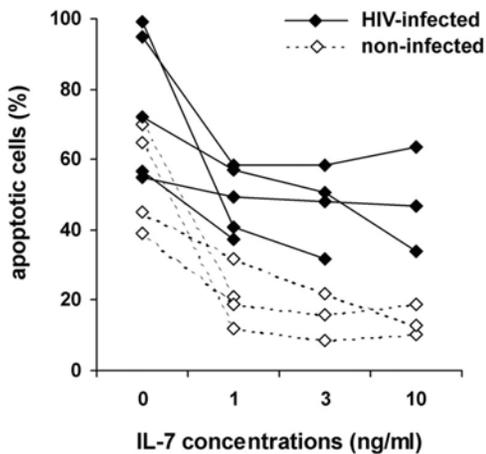


Figure 8. Effect of IL-7 on the survival of T cells isolated from HIV-1 infected or healthy donors. T cells were cultured in presence of various concentrations of IL-7 for 7 days and apoptosis was detected by Annexin-V staining.

We then looked further in vivo at the levels of IL-7 and amount of cells expressing IL-7R α in HIV-1 infected patients. Like in previously published work (Napolitano et al., 2001) (Fry et al., 2001) (Llano et al., 2001) (Mastroianni et al., 2001) we also found that the IL-7 level correlates with the amount of CD4+ T cells (Fig 9a). In addition we also observed a link between the level of IL-7 in serum and expression of its receptor. At higher concentrations of IL-7 there was a larger proportion of T cells lacking the IL-7R α (Fig 9b). This was interesting in respect to the possibility that IL-7 might regulate the expression of its own receptor. To verify this we cultured T cells from healthy donors with IL-7 and measured the expression of IL-7R α .

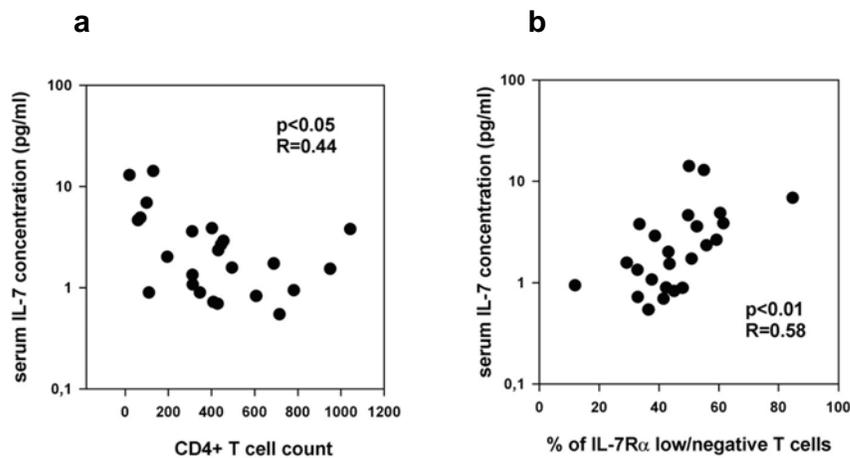


Figure 9. Correlation of serum IL-7 levels with the CD4+ T cell decline and reduced IL-7R α expression. The IL-7 concentration in the serum of HIV-1 infected patients is shown together with CD4+ T cell count (a) and the percentage of T cells that have lost the IL-7R α (b).

Upon these conditions, the receptor expression declined rapidly after 3 hours of incubation and remained low upon further incubation in IL-7. When IL-7 was washed out the receptor regained its expression starting directly after removal of the cytokine and reaching its full expression after 24 hours. This clearly indicates that IL-7 regulates the expression of its own receptor in cells obtained from healthy individuals. When T cells from HIV-1 infected patients with high levels of IL-7 in serum were put in culture without IL-7 no restoration of the IL-7R α expression was measured even when the cells were kept for as long as 5 days without IL-7. These findings indicate that during HIV-1 infection there is a permanent down-regulation of the IL-7R α not only caused by IL-7 itself. Thus,

the mechanism leading to down-regulation of the IL-7 receptor during HIV-1 infection needs further investigation.

The down-regulation of IL-7R α in HIV-1 infection is seen in both CD4+ and CD8+ T cells, and this does not reflect a fully activated T-cell phenotype since activation markers such as CD69, CD25 and CD70 are not increased on these cells. The early markers HLA-DR and CD38 are however expressed on these cells reflecting a previous activation stage.

One could speculate that the loss of IL-7R α seen would mirror the loss of naïve cells observed in HIV-1 infected patients, but this does not seem to be the case. We looked at different T cell populations using the markers CD45RA and CCR7 and compared their number between infected and non-HIV-1 infected individuals. In HIV-1 infected patients there were an increased number of CCR7- memory cells, and most of them lacked the IL-7R α . Cells of the CD45RA+ and CCR7+ naïve phenotype that had lost their receptor could be found in patients as well as in non-infected subjects. Thus the lack of the IL-7R α receptor does not simply reflect the loss of one cellular compartment, naïve or memory, during infection, but seem to occur in all differential stages of T cell subpopulations. Interestingly, the CD45RA+ CCR7- effector memory population in healthy individuals was coupled with a low frequency of the IL-7R α .

In view of the fact that the expression of CD28 is lost during HIV-1 infection (Effros et al., 1996), it was interesting to study the expression of IL-7R α in conjunction with CD28 loss. We found that T cells negative for CD28 were also low in IL-7R α expression, and this was true both for infected and uninfected subjects. In response to IL-7, CD28 negative cells had less expression of Bcl-2 as compared to CD28 positive cells. The low expression of CD28 is also seen in autoimmune diseases (Salomon and Bluestone, 2001) and in the elderly population (Nociari et al., 1999), which makes it interesting to compare HIV-1 infection to an immune system that is weak, exhausted and less able to restore an effective immune cell repertoire (Appay and Rowland-Jones, 2002).

3.1.3 IL-7 induced Fas and its implications in HIV-1 infection (III)

The levels of IL-7 during HIV infection are higher than in healthy individuals and it has also been observed that the expression of Fas and Fas-mediated apoptosis is increased on T cells in this setting. Therefore we sought to investigate if there is a relationship between the high levels of IL-7 and Fas, if IL-7 can modulate the expression of Fas, and if T cells from HIV-1 infected individuals become more susceptible to apoptosis in the presence of high levels of IL-7.

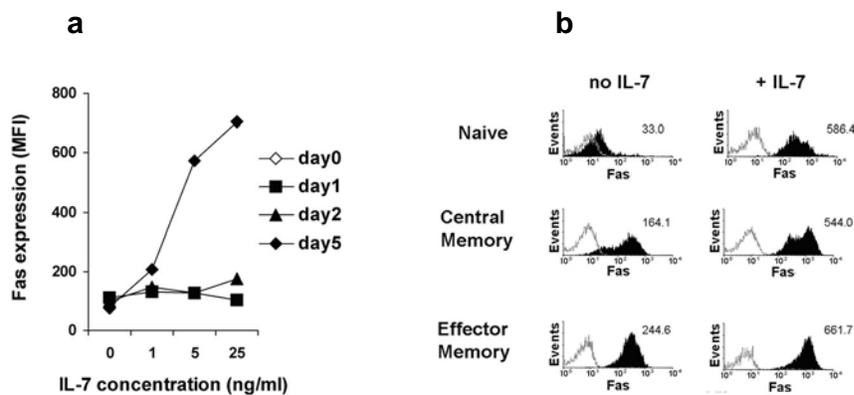


Figure 10. IL-7 induces up-regulation of Fas in 5 day cultures. a) Fas expression is measured after culture with different concentrations of IL-7 for 0, 1, 2 and 5 days. b) Fas expression is measured on naïve and memory cells incubated with or without 25ng/ml IL-7 for 5 days.

When culturing T cells from healthy donors with different concentrations of IL-7 we found a concentration dependent increase of Fas after 5 days of culture. This Fas up-regulation appeared gradually over time peaking at day 5, suggesting that in order to get a strong expression of Fas there needs to be a long-term exposure of T cells to IL-7(Fig 10a). The up-regulation of Fas was detected for both naïve and memory T cells and although the Fas expression in the memory cell compartment is already high in patients prior to IL-7 treatment, an increased expression can still be detected after IL-7 stimulation (Fig 10b).

When measuring Fas mRNA in IL-7 treated and untreated cells, we could not see an increased gene expression, meaning that the regulation of Fas must

occur at the post-transcriptional level. In fact, we hypothesize that Fas is transported to the surface from an intracellular storage due to IL-7 exposure. This hypothesis is based on the observation that Fas protein levels measured in the whole cell are equally high in IL-7 treated cells as compared to untreated cells.

Since there are other cytokines involved in the regulation of apoptosis, such as IL-2, IL-4 and IL-15, sharing the common γ -chain of the IL-7R, we looked at their ability to induce Fas up-regulation on T cells in a similar way as IL-7. IL-2 and IL-15 had a comparable effect on Fas up-regulation as seen for IL-7. IL-4 however, did not influence Fas regulation at all, arguing against the possibility that the common γ -chain using cytokines in general can control the expression of Fas (data not shown).

For Fas-mediated apoptosis to occur there needs to be a link between the Fas molecule to the actin skeleton via ezrin which gives the cells a polarized shape and an assembly of the Fas molecules to one or possibly two extending protrusions as described in the introduction (Fais et al., 2000) (Luciani et al., 2004). We cultured T cells from healthy donors with or without IL-7 for 5 days and then stained them for Fas and Ezrin in an immunocytochemical assay. We found that IL-7 induced a polarized shape of the cells and Fas and Ezrin were co-localized to the protrusions. This was observed both for naïve and memory cells cultured with IL-7, yet memory cells had a more profound polarization, as compared to untreated cells. As expected we also found a similar polarization of Fas molecules on anti-CD3 activated T cells.

It is also known that the surface molecule CD43, similarly to Fas, is recruited to the protrusions on activated T cells through association with Ezrin. Likewise we found that these molecules co-localized when we stained for CD43 and Ezrin on IL-7 treated cells. To further strengthen the finding of the co-localization of Fas and Ezrin occurring upon IL-7 stimulation, we performed fluorescence resonance energy transfer (FRET) analysis. A higher FRET efficiency was observed for IL-7 treated T cells as compared to untreated T cells. Also here anti-CD3 stimulated cells were used as positive control. These findings demonstrate that incubation with IL-7 renders T cells susceptible to apoptosis by inducing the association of Fas and Ezrin as described for long-term activated T cells (Parlato et al., 2000) (Fais et al., 2005).

The influence of IL-7 on Fas expression was tested *in vivo* in a monkey model where cynomolgus monkeys were injected daily for 10 days with IL-7 at different concentrations. Fas was measured before and after treatment, and there was a clear Fas up-regulation in both CD4+ (Fig 11a) and CD8+ (Fig 11b) T cells, most prominent at the highest concentration of IL-7, in these animals. When treatment was stopped the Fas levels decreased back to original levels again after 10 days. These results clearly reflect the biological relevance of the findings we obtained *in vitro*.

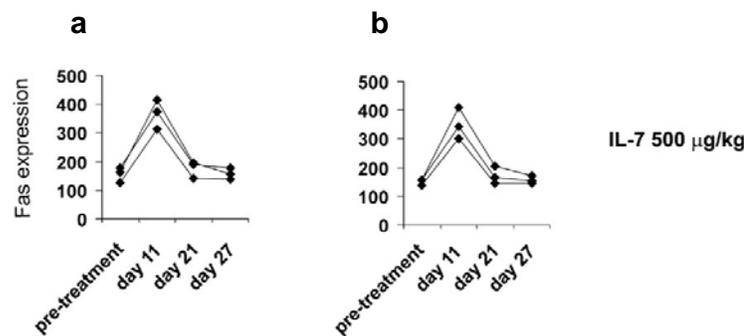


Figure 11. Fas expression is increased *in vivo* on T cells from monkeys injected with IL-7. a) Fas expression on CD4+ T cells. b) Fas expression on CD8+ T cells

The findings *in vitro* and in monkeys suggested the possibility that the high IL-7 levels found in HIV-1 infected patients also would have an influence on Fas expression and possibly also Fas-mediated apoptosis. For this purpose blood was collected from HIV-1 positive individuals and IL-7 levels in serum were compared to Fas expression levels and CD4+ T cell counts. In line with our hypothesis there was a significant correlation between Fas expression on T cells and IL-7 levels, and patients with high levels of IL-7 also presented high levels of Fas expression (Fig 12a). Looking more in depth at the different T cell populations, the comparison of Fas expression on the IL-7R α negative and positive T cells revealed that in cells lacking the IL-7R α there was no correlation between Fas expression and IL-7 levels (Fig 12c). The IL-7R α negative T cells had all a high level of Fas possibly indicating an activated state. In contrast, in T cells bearing the IL-7R α , and thus able to respond to IL-7, there was a correlation between the two parameters (Fig 12b). In the naïve and memory T cell populations, characterized by the expression of CD45RA

and IL-7R α , the same pattern was also seen. On effector cells CD45RA+ IL-7R α - and on the memory phenotype CD45RA- IL-7R α -, both lacking IL-7R α , Fas expression did not correlate with the level of IL-7.

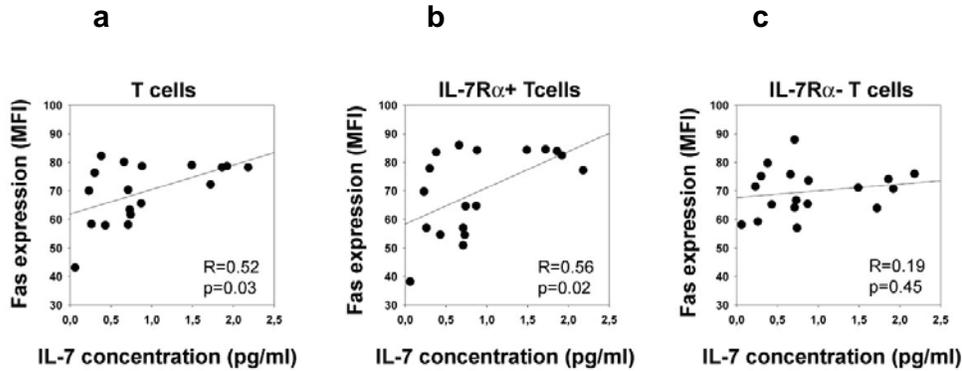


Figure 12. High Fas expression on T cells from HIV-1 infected patients is associated with increased levels of IL-7 in serum. Fas expression is measured on all T cells (a), IL-7R α positive T cells (b) and IL-7R α negative T cells (c).

The increased Fas expression that we find due to IL-7 stimulation of IL-7R α bearing T cells should render these cells more sensitive to Fas-mediated apoptosis. This was tested in an apoptosis assay where cells from healthy donors were cultured with or without IL-7 for 5 days and then stimulated with anti-Fas antibodies followed by annexin-V staining. These results clearly demonstrated that T cells, treated with IL-7 were much more sensitive to Fas-mediated apoptosis than untreated cells. There was no difference between CD4+ and CD8+ T cells, but cultures of memory cells had a higher percentage of apoptotic cells than naïve cells (Fig 13).

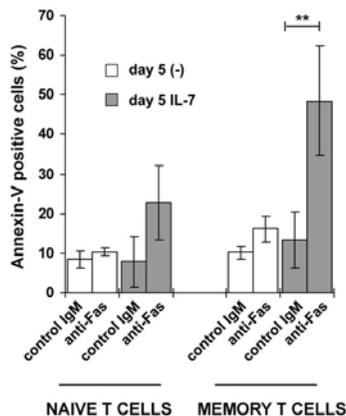


Figure 13. IL-7 sensitizes T cells for Fas-mediated apoptosis in vitro. Apoptosis was induced by anti-Fas antibodies in naïve and memory T cells after 5 days of incubation with IL-7.

The apoptosis sensitivity of IL-7 stimulated T cells was also verified on specimens obtained *ex vivo* from HIV-1 infected individuals. T cells from HIV-1 infected patients were cultured together with anti-Fas antibodies and, upon this condition, we found a correlation between the amount of apoptosis sensitivity both in naïve (Fig 14a) and memory (Fig 14b) T cells and levels of IL-7 in serum in these patients. When looking at the correlation between the expression of Fas and Fas-mediated apoptosis, a significant correlation could be seen only for the memory cell compartment. The naïve cells showed a non-significant but very strong trend towards a correlation for these two parameters ($p=0,07$).

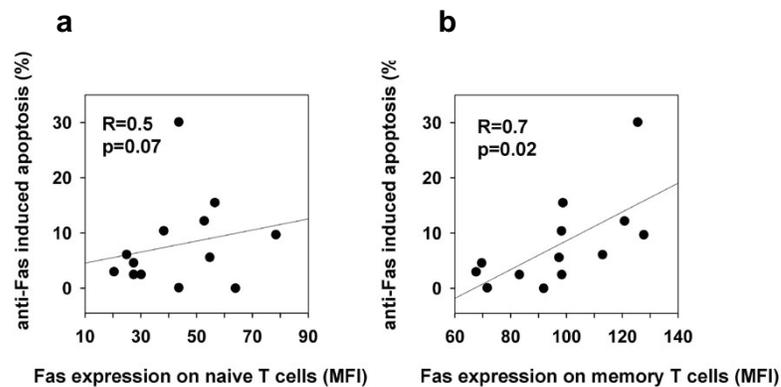


Figure 14. Correlation of Fas expression and Fas-mediated apoptosis from HIV-1 infected patients. Naïve and memory T cells were isolated from HIV-1 infected patients and Fas expression was measured. Apoptosis was induced using anti-Fas antibodies and measured by Annexin-V. Results from the naïve (a) and memory (b) T cells are shown.

3.2 BYSTANDER APOPTOSIS DURING CHRONIC IMMUNE ACTIVATION (IV)

During infection with *L. Major* uninfected keratinocytes are surrounded by inflammatory agents and killed through apoptosis via death receptor pathways such as Fas and TRAIL.

When supernatants from *in vitro* cultures of *L. Major* infected PBMCs were analyzed, higher levels of soluble FasL (Fig 15a) and TRAIL (Fig 15b) were found compared to non-infected control PBMC supernatants. In addition, the

levels of IFN- γ were also higher in infected cultures (results not shown) pointing towards an induction of an inflammatory process by the parasitic infection.

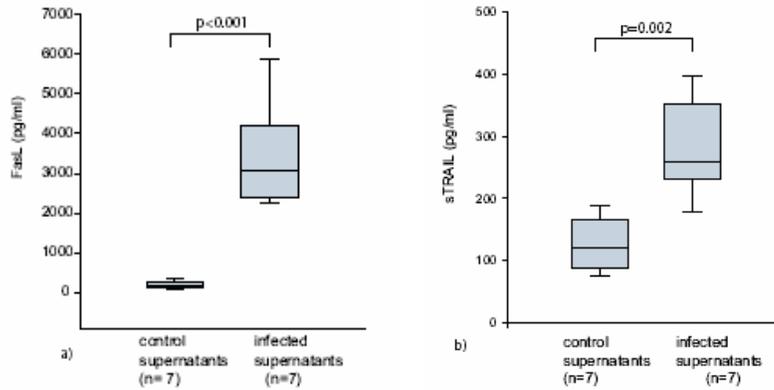


Figure 15. High levels of sFasL and sTRAIL were measured in supernatants from *L. Major* infected PBMCs. sFasL (a) and sTRAIL (b) were measured by ELISA in supernatants from *L. Major* infected PBMCs and uninfected control culture.

A closer look into the actual gene expression of the human keratinocyte cell line HaCaT, suggested that incubation with supernatants from infected PBMC cultures for 6 and 24 hours induced the expression of Fas, TRAIL and TRAIL-R2 on HaCaT (Fig 16). This was analyzed by microarray analysis where 96 genes belonging to several apoptotic pathways were included. To further investigate if the increased gene expression would reflect into increased expression on the surface, FACS analysis was applied on HaCaT exposed to infected supernatants for 20 hours. Upon these conditions there was an increased expression of Fas and TRAIL in concordance with the microarray findings.

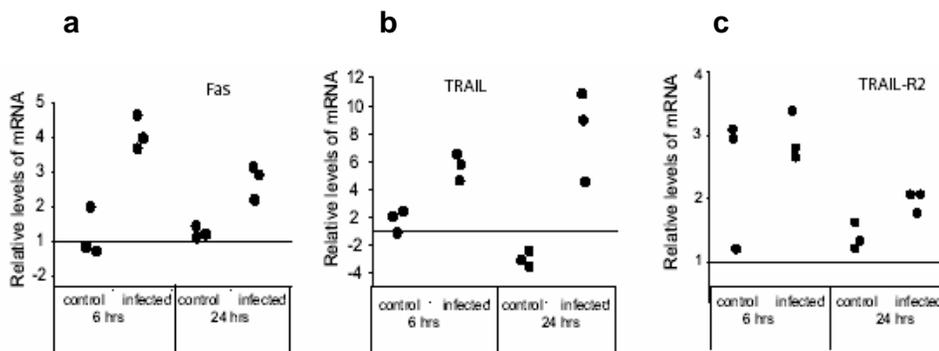


Figure 16. Changes of mRNA expression of Fas, TRAIL and TRAIL-R2 in HaCaT exposed to supernatants from *L. Major* infected PBMC cultures. HaCaT cells were incubated with

supernatants from three different uninfected or *L. Major* infected PBMC cultures for 6 or 24 hours and changes in mRNA expression were measured by microarray analysis as compared to untreated HaCaT cells.

The amounts of sTRAIL, already high in the supernatants, did not increase upon incubation with HaCaT, meaning that the keratinocytes did not seem to secrete any sTRAIL themselves.

The expression of the four TRAIL receptors on the keratinocytic cell line, detected by FACS analysis, did not change upon incubation with supernatants from monocyte cultures infected with *L. Major*, since all four receptors were expressed on untreated HaCaT cells, and their expression did not change significantly after exposure to *L. Major* infected supernatants. In a histological approach it was found that TRAIL-R1 and -R3 was predominantly expressed, and also TRAIL-R2 was weakly expressed on the surface of untreated HaCaT cells. TRAIL-R4 was only very weakly expressed on the surface of cells, but rather found in intracellular vesicles.

The expression of death receptors should presumably reflect the sensitivity to death receptor mediated apoptosis. In a previous study, where PBMC's from *L. Major* infected patients were reinfected *in vitro*, culture supernatants were shown to induce apoptosis in HaCaT (Eidsmo et al., 2005). Supernatants from PBMCs infected with *L. Major* also induced apoptosis in HaCaT (Fig 17a). Apoptosis was in addition induced in a similar way by the agonistic monoclonal Fas antibody CH11 (Fig 17b). The apoptosis of HaCaT cells induced by CH11 could be blocked up to 70% by the anti-Fas monoclonal antibody ZB4. Likewise, apoptosis of HaCaT cells induced by supernatants from *L. Major* could be blocked by ZB4, but only partly (40%). This suggests that other apoptotic pathways are involved apart from the Fas pathway. TRAIL-induced apoptosis was also investigated on the HaCaT cells by addition of recombinant TRAIL, and 90% of the induced apoptosis could be blocked by the TRAIL neutralizing antibody 2E5. There was no additive effect of the combination of monoclonals against FasL and TRAIL (Fig 17c).

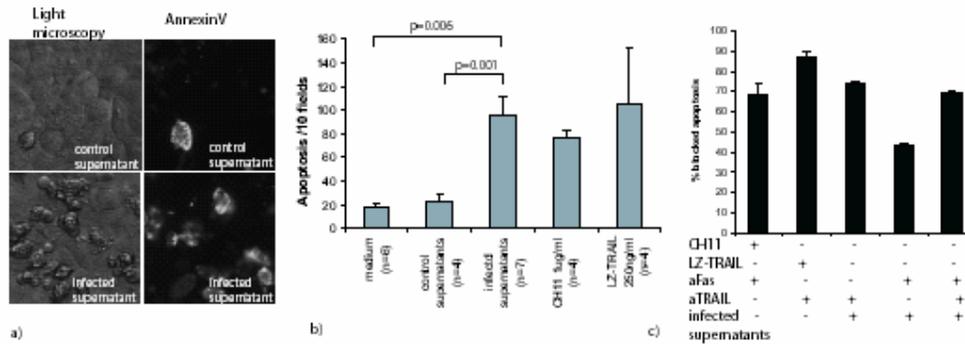


Figure 17. Supernatants of *L. Major* infected cultures analyzed for their capacity of inducing apoptosis of HaCaT cells. a) Cells analyzed by light microscopy for morphological changes characteristic of apoptosis. b) Apoptosis induction of HaCaT cells by supernatants from *L. Major* infected cultures as compared to FasL and TRAIL mimicking antibodies. c) % of apoptosis blocking by anti-Fas and anti-TRAIL antibodies.

Since the immortalized cell line HaCaT differs from primary keratinocytes in the expression of several apoptotic pathways we did similar experiments on human epidermal keratinocytes (HEKs). HEKs were exposed to supernatants from *L. Major* infected PBMCs and membrane expression of Fas, TRAIL and TRAIL-R1-4 was analyzed. Overall, Fas was expressed to a higher extent by HEK as compared to HaCaT, and the Fas expression was not increased when HEK were exposed to supernatants from *L. Major* infected PBMCs. TRAIL, on the other hand, had a similar expression by HEK as found in HaCaT cells, and was up-regulated when infected supernatants were added. The receptors TRAIL – R1-4 were all expressed on HEK, and TRAIL-R1 and R2 were partially down-regulated upon incubation with infected supernatants. The down-regulation possibly reflects that the receptors are occupied by their ligand TRAIL, or that the cells remaining in the cultures after incubation with infected supernatant (and induction of apoptosis) were low in TRAIL-R1 and –R2 expression.

Even though HEK presented a high expression of Fas, they were much more resistant to Fas-mediated apoptosis as compared to HaCaT cells (Fig 18a). On the contrary, despite the low expression of TRAIL on HEK cells, HEK were more susceptible to TRAIL-mediated apoptosis than HaCaT cells. Apoptosis in HEK was also induced by supernatants from *L. Major* infected PBMCs in a similar way as in HaCaT, although to a lower degree. The blocking experiments using anti-Fas and anti-TRAIL antibodies showed a partial block in apoptosis

(Fas 38%, and TRAIL 60%) and also here there was no additive effect of the antibodies (Fig 18b).

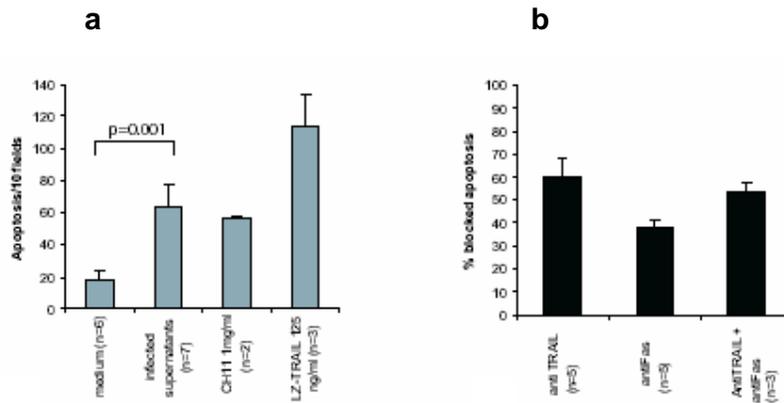


Figure 18. Human epidermal keratinocytes (HEK) were investigated for susceptibility to apoptosis through Fas and TRAIL receptors. Induction of apoptosis using FasL and TRAIL mimicking antibodies (a) and blocking of apoptosis by anti-Fas and anti-TRAIL antibodies (b) are shown.

In skin biopsies from patients infected with *L. Major* and healthy controls the expression of Fas, TRAIL and TRAIL-R1-4 was detected by immunohistochemistry. The biopsies from patients with Cutaneous Leishmaniasis (CL) were taken at the site of the ulcer and they were considered to be active or healing as previously described (Eidsmo et al., 2005). Both Fas and TRAIL expression was increased in CL skin as compared to healthy controls, although there were some individual differences in the expression between subjects. The TRAIL receptors were also investigated and we found that TRAIL-R1 was not expressed in CL skin ulcers, but TRAIL-R2 expression was stronger in CL skin as compared to controls that instead had a weak TRAIL-R1 expression. TRAIL-R3 was found to be expressed in both healthy and diseased skin and TRAIL-R4 was only weakly detected in CL skin and not at all in control. The up-regulation of TRAIL-R2 and TRAIL on CL skin suggests the presence of a pro-apoptotic environment since the TRAIL-R2 signaling has been said to be more efficient in apoptosis induction as compared to TRAIL-R1 (Kelley et al., 2005).

3.3 CONCLUSIONS AND FUTURE PERSPECTIVES

The impact of IL-7 in HIV-1 infection is complex and this cytokine seems to act selectively at different stages of disease linked to HIV-1 infection. Early during primary infection, higher levels of IL-7 in serum appear to be an advantage for the restoration of T cell numbers when ART is introduced. During chronic infection there is a correlation between IL-7 and CD4+ T cell count where IL-7 is higher in patients with low CD4+ T cell count, and this is thought to represent an attempt to homeostatic regulation of T cell numbers. However, this does not appear to be a general rule in HIV-1 infection since the ratio between their CD4+ and CD8+ T cell counts and the levels of IL-7 varied in some non-treated individuals included in a small size longitudinal study. In LTNPs we found that high levels of IL-7 could be predictive of disease progression. This is of course a very interesting finding, and it can be speculated that this may be due to the fact that the T cells have become non responsive to IL-7 and thus can not get enough survival signals. In addition, the high IL-7 levels might reflect the diminished use of the cytokine that instead accumulates in the body fluids and this scenario requests further studies to be confirmed. There is also the possibility that IL-7 plays a role in promoting the replication of CXCR4 using viruses often seen at the late stages of disease. It has been shown that the interaction of thymic epithelial cells (TEC) with CD4+ T cells in culture induces an up-regulation of CXCR4 expression. TEC secretes IL-7, which increases cell surface expression of CXCR4 and efficiently overcomes the down-regulation induced by SDF-1 alpha, also produced by TEC. IL-7 also strengthens uptake of the virus via CXCR4 co-receptor, by leading to actin polymerisation, a process necessary for virus entry (Schmitt et al., 2003).

The lack of response to IL-7 during HIV-1 is probably due to down-regulation of the IL-7R α . We show that IL-7 down-regulates its own receptor on T cells in vitro and in HIV-1 infected patients there is a correlation between the IL-7 levels in serum and the expression of the IL-7R α on T cells where high IL-7 levels are associated with low expression of the receptor. In HIV-1 infection however, in addition to the high levels of IL-7, there might be other mechanisms that cause IL-7R α down-regulation. This is suggested by the finding that the receptor expression is not restored when IL-7 is removed from

ex vivo cultures of T cells obtained from patients, on the contrary of what is seen for healthy individuals. This may be another explanation for the LTNPs that progress to symptomatic HIV-1 infection in spite of the high levels of circulating IL-7, as their T cells might have developed a permanent defect in the expression of the IL-7R α . In this direction Sasson and collaborators (Sasson et al., 2006b) found that an increased proportion of CD4+ IL-7R α - and CD8+ IL-7R α - T cells could be found in peripheral blood of LTNP's as compared to healthy volunteers. It would be interesting to study whether IL-7R α would be re-expressed on T cells upon ART therapy, and if renewed IL-7R α expression can replenish a somewhat functional T cell pool.

Chronic immune activation present in HIV-1 and *L. Major* infections leads to a state where death receptors are up-regulated, and in particular uninfected bystander cells die through death receptor-mediated apoptosis. This phenomenon is thought to occur as a natural occurring process aiming at terminating an immune response when the antigen is cleared. However, in a chronic infection there is a constant antigen triggering and no resolution of the infection. In the case of HIV-1 infection, according to our study, IL-7 among others seems to be one important player in the increased expression of Fas. When this occurs in an environment where high amounts of FasL are present in the circulation there is obviously the risk for higher incidence of Fas-mediated apoptosis.

The increased expression of Fas caused by IL-7 might represent a physiological mechanism acting as some sort of security switch against an uncontrolled cell division likewise that observed for activated cells. IL-7 treated cells are however not really activated since they do not express several activation markers, apart from some early ones indicating a semi-activated state. High IL-7 levels in HIV-1 infection may be detrimental in the presence of high expression of FasL where there may be an increased triggering of apoptosis on the T cells expressing high levels of Fas. This harmful scenario needs to be further evaluated as it has been discussed to use IL-7 immunotherapy in HIV-1 infected patients to increase the T cell pool. Perhaps, IL-7 immunotherapy would be beneficial in early HIV-1 infection when high IL-7

levels seem to be of benefit for the restoration of T cells upon ART therapy. However although it is well characterized that FasL expression is up-regulated during chronic HIV-1 infection (Medrano et al., 1998) (Silvestris et al., 1998), it is yet not well studied if FasL dysregulation occurs already during primary infection.

In the context of IL-7, anti-IL-7 antibodies could be discussed as a way of inhibiting the increased expression of Fas, and thus diminish the sensitivity to apoptosis. The problem with this is that IL-7 is a survival factor for naïve and memory cells and cannot be fully replaced by other cytokines like IL-15. In an ideal scenario, it would be important to learn how to modulate the expression of Fas and FasL with the purpose of reducing apoptosis during HIV-1 infection. This is partially, but not completely, achieved during treatment with ART and additional drugs should possibly be developed to reduce immune activation during HIV-1 infection. We did not look at TRAIL expression as a response to IL-7 in our studies, but this would obviously also be interesting since TRAIL has been shown to be up-regulated and to be involved in CD4+ T cell apoptosis in HIV-1 infection as well (Herbeuval et al., 2005a) (Herbeuval et al., 2005b).

We found an increased Fas, TRAIL-R2 and TRAIL expression on uninfected keratinocytes upon exposure to supernatant from *L. Major* infected cultures and in histological immunostainings of skin specimens from patients with Cutaneous Leishmaniasis. This up-regulation of death receptors on keratinocytes appears to be due to the inflammatory milieu in close vicinity to the cells. The expression of death receptors also renders keratinocytes more sensitive to apoptosis and, *in vivo*, they probably die through bystander effect due to infiltrating immune cells expressing death ligands. Thus, the keratinocytes are either killed by soluble FasL or TRAIL, or by membrane bound death receptors on surrounding circulating cells including other activated keratinocytes. Blocking Fas and TRAIL pathways *in vitro* inhibits the majority of apoptosis occurring in these experimental systems but not all, thus leaving open the possibility that other mechanisms and pathways may be involved in this process.

In summary this work contributes to the understanding of the role of chronic immune activation in some aspects of pathogenesis during HIV-1 infection. The findings on the contribution of high IL-7 levels to Fas up-regulation and increased sensitivity for Fas-mediated apoptosis brings forward concerns on the use of IL-7 immunotherapy for the treatment of HIV-1 infection. Obviously our new results generate several interesting questions to be addressed: as an example it can be mentioned that further investigation on the mechanism behind the down-regulation of IL-7R α in HIV-1 infection is needed and is in progress. This is of relevance in that it may represent a point of attack for new therapy intervention during HIV-1 infection. The memory T cell pool needs to be maintained for an effective immune response to occur and thus to modulate the re-expression of IL-7R α is of utmost importance for control of virus replication.

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